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US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDR-P
3100 Ricketts Point Road
Aberdeen Proving Ground, MD
21010-5400

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RESEARCH ARTICLE

Stimulation of central A1 adenosine receptors suppresses seizure and neuropathology in a soman nerve agent seizure rat model

Thaddeus P. Thomas[1,2] and Tsung-Ming Shih[1]

[1]US Army Medical Research Institute of Chemical Defense and [2]US Army Research Laboratory, Aberdeen Proving Ground, MD, USA

Abstract

The current regimen for treating nerve agent poisoning does not sufficiently suppress the excitotoxic activity that causes severe brain damage, especially in cases where treatment is delayed and nerve agent-induced status epilepticus develops. New therapeutic targets are required to improve survivability and minimize neuropathology after irreversible acetylcholinesterase inactivation. Earlier studies have shown that systemic delivery of adenosine agonists decreases nerve agent lethality; however, the mechanism of protection remains to be understood. The primary aim of this study was to investigate the role of central adenosine receptor (AR) stimulation in neuroprotection by directly injecting (6)-cyclopentyladenosine (CPA), an adenosine agonist specific to the A1 receptor subtype (A1R), into the brain intracerebroventricularly (ICV) in a soman seizure rat model. In addition to general A1R stimulation, we hypothesized that bilateral micro-injection of CPA into the cholinergic basal forebrain (BF) could also suppress excitotoxic activity. The results from these studies demonstrated that centrally administered adenosine agonists are anti-seizure and neuroprotective. CPA-delivered ICV prevented seizure and convulsion in 100% of the animals. Moreover, neuropathological evaluation indicated that adenosine treatments reduced brain damage from severe to minimal. Inhibition of the BF via CPA had varied results. Some animals were protected by treatment; however, others displayed similar pathology to the control. Overall, these data suggest that stimulating central ARs could be an effective target for the next generation countermeasures for nerve agent intoxication.

Keywords

Adenosine agonist, anti-seizure, nerve agent, neuroprotection

History

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Introduction

Organophosphorus (OP) chemical warfare nerve agents (CWNAs), such as soman and sarin, irreversibly inhibit acetylcholinesterase (AChE), the enzyme responsible for hydrolyzing the neurotransmitter acetylcholine (ACh) in the cholinergic synapses and neuromuscular junctions (McDonough & Shih, 1997). Following exposure to CWNAs, the earliest neurochemical events detectable in the central nervous system (CNS) are the inhibition of AChE and an immediate increase in brain neurotransmitter acetylcholine (ACh) levels (Shih, 1982). After a longer duration of seizure activity, changes in the levels of excitatory (glutamate) and inhibitory (γ-aminobutyric acid, GABA) amino acid transmitters are observed (el-Etri et al., 1992; Fosbraey et al., 1990; Lallement et al., 1991; O'Donnell et al., 2010, 2011; Wade et al., 1987). Many potential inhibitory compounds and drugs along these lines of neurotransmission perturbations have been investigated (McDonough & Shih, 1997; Shih, 1990; Shih et al., 2003). These treatments have limited efficacy in protecting the CNS, particularly in cases of prolonged seizure activity. Therefore, investigation and exploration of new therapeutic targets for CWNA countermeasures are needed.

Adenosine is an endogenous substance that regulates multiple peripheral and central physiologic functions. It is released during normal metabolic activity into the extracellular space where it acts on adenosine receptors (ARs) (Ribeiro et al., 2002). Adenosine modulates cellular activity by stimulating specific AR subtypes that are classified according to their effect on adenylyl cyclase; A1 inhibits activity via Gαi proteins (Haas & Selbach, 2000; Sperlagh & Vizi, 2011; St Hilaire et al., 2009). Adenosine's most notable physiologic effect occurs with the stimulation of A1Rs in the brain. Activation of central A1Rs elicits a profound inhibitory effect on neuronal excitability and synaptic transmission.

Address for correspondence: Tsung-Ming Shih, US Army Medical Research Institute of Chemical Defense, ATTN: MCMR-CDR-P (Dr. T.-M. Shih), 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010-5400, USA. Tel: +1 410 436 3414. Fax: +1 410 436 2072. E-mail: tsungming.a.shih.civ@mail.mil

Abbreviations

ACh, acetylcholine;
AChE, acetylcholinesterase;
AR, adenosine receptor;
AMN, atropine methylnitrate;
BF, basal forebrain;
BBB, blood–brain barrier;
CWNA, chemical warfare nerve agent;
CNS, central nervous system;
GABA, γ-aminobutyric acid;
CPA, (6)-cyclopentyladenosine;
ICV, intracerebroventricular;
IM, intramuscular;
IP, intraperitoneal;
LV, lateral ventricle;
MTD, maximum-tolerated dose;
NMDA, *N*-methyl-D-aspartate;
OP, organophosphorus compound;
SC, subcutaneous.

Pre-synaptically, adenosine reduces the influx of calcium, which suppresses the release of glutamate. Post-synaptically, adenosine decreases neuronal excitability by inhibiting *N*-methyl-D-aspartate (NMDA) receptors and voltage-sensitive calcium channels (Malva et al., 2003; Ribeiro et al., 2002). A1Rs are distributed throughout numerous brain structures including the cortex and thalamus, and have the highest densities in critical cholinergic centers, such as the basal forebrain (BF), hippocampus, and striatum (Bjorness & Greene, 2009; Svenningsson et al., 1997). In addition to the brain, A1Rs are also widely distributed throughout the periphery. A1Rs have been detected in the heart, aorta, liver, kidney, eye, and bladder (Dixon et al., 1996). In the periphery, A1R stimulation primarily decreases heart rate and blood pressure.

Previous research has shown that exogenously administered adenosine provides neuroprotection from various trauma including epilepsy, hypoxia, and ischemia (Basheer et al., 2004; Cunha, 2005; Lynge & Hellsten, 2000; Schubert et al., 1997; Svenningsson et al., 1997; van Helden et al., 1998; Wardas, 2002). These earlier data suggest that adenosine's protective mechanism involves the partial neutralization of neuronal Ca^{++} overload that leads to cell death (Schubert et al., 1997). Adenosine's inhibitory effect on neuronal excitability has also been exploited for the treatment of drug-resistant epilepsy (Gouder et al., 2003; Huber et al., 2002; Young & Dragunow, 1994). Unfortunately, adenosine therapeutics have not been widely implemented because of the profound reductions in the heart rate and blood pressure that peripheral AR stimulation triggers (Biaggioni, 1992; Dunwiddie & Masino, 2001; Schindler et al., 2005). Despite such cardiovascular effects, van Helden et al. (1998) recognized adenosine's potential as a CWNA countermeasure. In their early study, the A1 adenosine agonist (6)-cyclopentyladenosine (CPA) was shown to reduce nerve agent lethality; intramuscular (IM) injections of CPA decreased extracellular ACh levels, diminished seizure activity, and improved survivability in a soman-induced seizure rat model. Other researchers, many of whom are affiliated with van Helden, pursued adenosine in nerve agent models, and identified its neuroprotective properties (Bueters et al., 2002, 2003; Compton, 2004; Joosen et al., 2004; Tuovinen, 2004). However, the mechanism of protection has yet to be agreed upon. Much of the contention can be attributed to the systemic administration method. Since adenosine agonists were injected intramuscularly or subcutaneously, both central and peripheral receptors were stimulated. Consequently, marked decreases in heart rate and blood pressure accompanied the suppression of CNS hyperactivity. Some have concluded that this concomitant decrease in cardiac output protected the brain because very few nerve agents likely circulated to the brain and preserved central AChE activity (Joosen et al., 2004). The results from Bueters et al. (2003) study supported that hypothesis as AChE activity was greater in CPA-treated animals than controls (Bueters et al., 2003). If a reduction in cardiac output was in fact the mechanism of CPA's protection, then similar effects would be expected in VX-exposed animals. However, Bueters et al. (2002) found that CPA at similar doses did not prevent pathologic ACh accumulation after VX exposure as it did against G agents; CPA only delayed the onset of symptoms. That delay could have been a result of AR-induced inhibition of neurotransmitter release and not from concomitant cardiovascular effects. While it is true that a peripherally AR-induced decline in circulation may delay the arrival of nerve agent to the brain, it does not fully explain CPA's observed protective benefits. The mechanism of nerve agent protection may be multi-factorial with the suppression of synaptic transmission and inhibition of neuronal activity playing a significant role (Cunha, 2005; Harrison et al., 2003; Joosen & van Helden, 2007; Wardas, 2002). To better understand adenosine's neuroprotective mechanism and to assess its true efficacy, it is essential that the effects of central and peripheral AR stimulations are separated. Therefore, the main goal for this project was to measure the neuroprotection offered by stimulating central ARs by microinjecting adenosine agonists directly into the brain's ventricular system.

In addition to assessing the neuroprotection that diffuse central AR stimulation offers, this project investigated for the first time if adenosinergic manipulation of the BF could minimize nerve agent-induced excitotoxic activity. *In vivo* models using electrical and chemical stimuli confirm that the BF is a critical center that promotes wakefulness, and its inhibition decreases brain activity (Lin et al., 2011). Furthermore, the BF excites numerous brain regions; its cholinergic neurons synapse on vital structures such as cerebral cortex, hippocampus, thalamus, amygdala, and olfactory bulb (Semba, 2000). Hyperactive ACh release from BF terminals may, therefore, be a major promoter of seizure activity. Since approximately 90% of the neurons in the basalis of Meynert are cholinergic and provide the principal source of ACh to the entire cortical surface (Mesulam et al., 1983), and adenosine has been shown to suppress neuronal discharge in the cholinergic BF (Strecker et al., 2000), we hypothesize that BF A1R stimulation may attenuate seizure activity after nerve agent exposure.

Methods

Subjects/animals

Male Sprague–Dawley rats weighing 250–350 g were purchased from Charles River Labs (Kingston, NY) and were individually housed at $21 \pm 2\,^{\circ}C$ and $50 \pm 10\%$ humidity with a 12-h light–dark schedule (with lights on at 06:00 h). Laboratory rodent chow and filtered tap water were freely available whenever the animals were in home cages.

Surgery

Using aseptic surgical techniques, animals were prepared first with the insertion of an electronic temperature ID transponder between the shoulder blades subcutaneously (Bio Medic Data Systems Inc., Seaford, DE) and then had wire-electrodes implanted into the skull for recording brain electroencephalographic (EEG) activity. A stereotaxic frame with computer-assisted guidance (Leica Microsystems Inc., Buffalo Grove, IL) was then used to drill two holes into skull and insert 26 gauge cannulae: (1) bilaterally toward the lateral ventricles (LVs) [Atlas Coordinates mm (AP, DV, L) (0.0, −4.5, ±1.5)] or (2) bilaterally toward the BF [(−0.35, −8.5, ±2.0)] (Paxinos & Watson, 2009) for drug administration. The rats were allowed to recover for 7 days before experimentation.

Soman seizure rat model

The soman-induced seizure rat model developed at the US Army Medical Research Institute of Chemical Defense (USAMRICD) for nerve agent-related neuroprotection studies was used for this study (Shih, 1990; Shih et al., 1991). This model began with pre-treating animals with 125 mg/kg HI-6 (1-2-hydroxyiminomethyl-1-pyridino-3-(4-carbamoyl-1-pyridino -2-oxapropane dichloride) intraperitoneally (IP). Thirty minutes after HI-6 pretreatment, animals were challenged with a subcutaneous (SC) injection of $1.6 \times LD_{50}$ (180 μg/kg) soman, a dose that produces 100% seizure and convulsion. One minute later, animals were injected IM with 2 mg/kg atropine methylnitrate (AMN) and treated with microinjections of the adenosine A1 agonist CPA into the LVs or BF. The HI-6 and AMN injections were incorporated into this model to mitigate soman's peripheral effects and promote 24-h survivability; seizure activity and neuropathology are not affected. CPA was purchased from Tocris Bioscience (Bristol, England). Soman was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, Aberdeen, MD). HI-6 was purchased from Phoenix Chemical Inc. (Bromborough, England), and AMN was purchased from Wedgewood Pharmacy (Swedesboro, NJ).

The rat's brain activity was recorded from EEG and used to detect seizure onset. The electrodes implanted into the rat's skull were connected to recording leads via a connecting plug that attached to the rat's head with dental cement. At the time of experiment, the rats were placed in individual recording chambers ($43 \times 30 \times 25$ cm) where they were able to move freely. Twenty-four hours after exposure, the animals were placed again in these chambers and recorded for an additional 30 min. The EEG data were collected from the CDE 1902 amplifiers and analyzed using Spike2 software (Cambridge Electronic Design, Ltd., Cambridge, England) and custom written MATLAB code. EEG data were continuously assessed by a trained technician who rated the seizure activity as absent or present.

Assessment of neuropathology

Once the *in vivo* segment of the experiment was completed, the rats were anesthetized with sodium pentobarbital-based euthanasia solution and perfused transcardially with saline followed by 4% paraformaldehyde in PBS. The brain was then extracted and stored in paraformaldehyde. This study implemented two different protocols for histology. The first protocol was used for the maximum tolerated dose (MTD) experiments. These brains were sectioned coronally at 50 μm through the cannulae implantation site, Nissl stained, and then analyzed by a trained pathologist to verify the accuracy of cannulae placement and evaluate toxicity. The second protocol was used for assessing neuropathology in the subsequent soman seizure experiments. Those brains were serial sectioned at 5 μm, stained with hematoxylin and eosin (H&E), and evaluated for neuropathology using established methodology (McDonough et al., 1995). A trained pathologist, who was unaware of treatment paradigm, analyzed and scored four brain regions: the piriform cortex, the thalamus, the dorsal and ventral hippocampus, using the standard rubric: 0 = no lesion; 1 = minimal (1–10%); 2 = mild (11–25%); 3 = moderate (26–45%); 4 = severe (>45%). To further stratify the data and obtain a more comprehensive measure of brain damage, a total score was calculated by summing the four regional scores. A total score of 16 indicates widespread severe damage.

Determination of MTD

Since it was believed that optimal neuroprotection would be achieved at the peak of AR stimulation, the first objective was to determine the MTD of CPA that produced no toxic side-effects when delivered ICV and to the BF. The primary adverse side-effect was expected to be peripheral AR stimulation-induced cardiovascular depression. To detect such a reaction, a pulse oximeter was initially implemented for non-invasively detecting changes in heart rate and oxygenation. However, limitations intrinsic to such a device prevented the acquisition of reliable data. Therefore, alternative methods for detecting toxicity were developed. A dose level was deemed intolerable if any of the following occurred: (1) the mucus membranes, ears, eyes, nose, feet, lips, and tails became cyanotic; (2) respiration was severely depressed; (3) animal did not recover within 24 h; or (4) death occurred.

The MTDs were determined in two parallel dose–response experiments, one for each injection site (the LV and BF), as shown in Table 1. Initial doses were chosen based on the data from previously published experiments that elicited neural inhibition and anticonvulsant effects with adenosine agonists (Anderson et al., 1994; Benington et al., 1995; Methippara et al., 2005; Thakkar et al., 2003; Yildirim & Marangoz, 2007). Four sets of rats were tested in sequential order at each injection site with three rats in each set. Every rat experienced two testing sessions separated by 24 h. CPA was bilaterally injected at a specified dose on day 1 at lower dose. On day 2, the animal received the same drug at an elevated dose.

For group 1 testing LV's MTD, the total dose of CPA was buffered in 10 µl of multisol (48.5% H_2O, 40% propylene glycol, 10% ethanol, and 1.5% benzyl alcohol) and administered bilaterally at a rate of 5 µl/min. For group 2 testing the BF's MTD, CPA was buffered in 2 µl of multisol and administered bilaterally at a rate of 1 µl/min. Multisol's safety and non-toxicity at the prescribed volumes and injection methods were verified in a separate experiment, in which two groups of six rats were injected with 10 µl of multisol to the LV and 2 µl of multisol to the BF. Physiologic and histological analysis determined these vehicle parameters to be safe and non-toxic.

After CPA injection, brain EEG activity was recorded for 5 h, and the behavioral response to adenosine was assessed using a modified functional observation battery (FOB) (Appendix) and a toxic sign test (Table 2). The FOB is a widely used method for assessing pharmacologic reactions; it measures mobility and the overall level of arousal/awareness (Bowen & Balster, 1997; Shih et al., 2006; Youssef & Santi, 1997). The toxic sign test detects more pathophysiologic signs such as tremors, convulsion, salivation, and uncoordinated movement. Twenty-four hours after the second injection, the rat was euthanized. The optimal MTD was determined to be the minimum dose of CPA that consistently produced maximum neural inhibition for each injection site.

The identified MTDs were then each tested in 12 additional rats to verify that a single injection per animal produced central effects without toxicity. Two groups of six animals had the MTDs microinjected as follows: (1) CPA into the LV and (2) CPA into the BF. The physiologic and behavioral responses were recorded for 5 h after injection, the animals were then returned to standard husbandry. Twenty-four hours after microinjection, animals were deeply sedated, perfused, and histologically prepared for pathological assessment. The MTD was verified to be non-toxic, i.e., no neuronal damage beyond what is to be expected from cannulae implantation.

General stimulation of A1Rs via CPA microinjection into the lateral ventricles

The neuroprotection offered by widespread central AR stimulation was investigated in the soman seizure rat model after MTD determination. The treatment target sites with atlas coordinates and administration regimens are summarized in Table 3. For LVs (Table 3, row 1), 24 animals were tested in two groups of 12. One minute after soman exposure, group 1 received an IM injection of AMN (2 mg/kg) and an intracerebroventricular (ICV) injection of CPA (700 µg; diluted in 10 µl of multisol) at a rate of 5 µl/min. Group 2 also received AMN (2 mg/kg, IM) 1 min after soman exposure, but was injected with multisol instead of CPA to serve as a control. At 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 30-min increments, behavioral responses were assessed using the FOB (Appendix), and toxic sign scores (Table 2) were recorded for a total of 5 h after soman exposure. EEG was continuously recorded during the 5-h observation period. Twenty-four hours later, an additional 30 min of EEG data were recorded for measuring final brain activity. Rats were then deeply anesthetized, euthanized by exsanguination, and histologically prepared for analysis of neuroprotection efficacy.

Focal stimulation of BF via A1Rs

The aim for this experiment was to determine if A1R-mediated inhibition of the cholinergic BF would attenuate nerve agent-induced excitotoxicity. Testing was performed on two groups of 12 animals that had cannulae implanted bilaterally in the BF (Table 3, row 2). One minute after soman exposure, both groups were injected with AMN (2 mg/kg, IM). Group 1 then received a microinjection of CPA (350 µg; diluted in 2 µl of multisol) into the BF at a rate of 1.0 µl/min. Group 2 received multisol instead of CPA to serve as a control. EEG was continuously recorded. At 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 30-min increments, behavioral FOB and toxic sign scores were recorded for a total of 5 h after soman exposure. Twenty-four hours later, following a final 30 min of EEG recording, animals were deeply anesthetized, euthanized, perfused, and histologically prepared for analysis of neuroprotection efficacy.

Data analysis

The Anderson–Darling normality test was used to determine whether the dataset would be analyzed with a parametric or non-parametric test. Regional differences in pathology severity (i.e., normal, minimal, mild, moderate, or severe) between treatment and control groups were compared using the

Table 2. Toxic sign scoring system.

Toxic signs scores	
Motor	0 = Normal, 1 = fasciculation's, 2 = tremors, 3 = convulsions
General	0 = Normal, 1 = mildly uncoordinated, 2 = impaired movement, 3 = prostrated
Salivation	0 = Normal, 1 = salivation
Lacrimation	0 = Normal, 1 = lacrimation
Eye	0 = Normal, 1 = nystagmus

Toxic signs were continuously scored following drug administration during the 5-h observation period on the day of the experiment and also scored again at the 24-h time point.

Table 1. Dose parameters for determining the maximum tolerated dose (MTD) for each injection site.

Group	Agonist	Animal set 1 ($N = 6$) (µg)		Animal set 2 ($N = 6$) (µg)		Animal set 3 ($N = 6$) (µg)		Animal set 4 ($N = 6$) (µg)	
1: LVs	CPA	5	150	290	430	575	700	850	1000
2: BF	CPA	1	70	150	215	290	350	430	500

The MTDs for CPA were determined in a dose escalation process for each brain target: the lateral ventricles (LVs) and basal forebrain (BF). There were four sets of animal per brain location with three animals per set. Each set of animals received one lower and one higher dose of CPA separated by 24 h. The animals were continuously monitored for signs of toxicity for 5 h and then again at 24 h after a second treatment. The dose escalation process stopped once toxicity was detected or the degree of physiologic response did not change.

Table 3. Description of adenosine treatment targets and administration regimens.

Target site	# of Animals	Injection location, atlas coordinates mm (AP, DV, L)	Treatment	Total volume (μl)	Injection rate (μl/min)
LVs	2 groups of 12	(0.0, −4.5, ±1.5)	700 μg CPA or multisol	10	5.0
BF	2 groups of 12	(−0.35, −8.5, ±2.0)	350 μg CPA or multisol	2	1.0

The maximum tolerated doses of CPA for the lateral ventricles (LVs) and the basal forebrain (BF) were microinjected into groups of 12 rats as a treatment to prevent seizure and neuropathology 1 min after a $1.6 \times LD_{50}$ dose (180 μg/kg) of soman. Results for the treatment groups were compared with control groups that received multisol vehicle instead of an adenosine agonist.

Chi-square test. The total neuropathology scores (0 = normal, 16 = severe) for each treatment group were compared to their controls using the Mann–Whitney test, and compared between treatment groups using the Kruskal–Wallis test. Rates of seizure prevention and survival were compared using Fisher's exact test. Comparison of the time to seizure and death were done using the Kaplan–Meier survival analysis and log rank test. Differences between treatment and their controls for body temperature were analyzed using the unpaired two-tailed *t* test. Statistical differences in the severity of toxic motor signs (fasciculation, tremor, and convulsion) between treatment and control groups were detected using a generalized linear model. The level of significance was set at $p < 0.05$.

Results

Determination of central MTD

The animals responded to the injections of CPA with a significant reduction in the central activity in a dose-dependent manner. When CPA was administered to the LVs and BF at the lower doses (150 μg for LVs and BF), the animals became lethargic and did not move spontaneously soon after injection. Doses below those values did not elicit notable reactions. As the doses increased, the animals entered a deeper state of sedation to the point where they could not be aroused for the duration of the 5-h observation period (>430 μg for LVs and >150 μg for the BF). Inspection of the EEG data demonstrated that their desynchronous baseline brain activity became more synchronized with lower frequencies and higher amplitudes (shown in Figure 1), similar to that of a deep sleep.

The minimum dose that produced the maximum response was determined to be the MTD for each administration level. This occurred at 700 μg CPA for the LVs group and 350 μg CPA for the BF group. At these doses, the LVs and BF groups experienced a statistically significant decrease in body temperatures during the 5-h observation period ($p < 0.05$). Whereas the animals that received only multisol decreased 1.4 ± 1.1 °C on an average, a likely product of normal circadian variations in temperature, the LVs group decreased 4.7 ± 2.2 °C, and the BF group decreased 3.3 ± 0.6 °C.

The latency between microinjection and behavioral effects shortened as doses escalated to MTD levels. Doses at MTD into the LVs produced maximum inhibition of behavioral and neural activity within 4–8 min and between 8 and 15 min for the BF MTDs. Recovery of central and motor functions varied between treatment regimens. The injection of CPA to the LVs resulted in the long lasting suppression of neural activity; the animals could not be aroused at the final 5-h assessment time point, but were able to recover by the next morning (>24 h). The effects of CPA at MTD to the BF began to diminish by the end of the 5-h period.

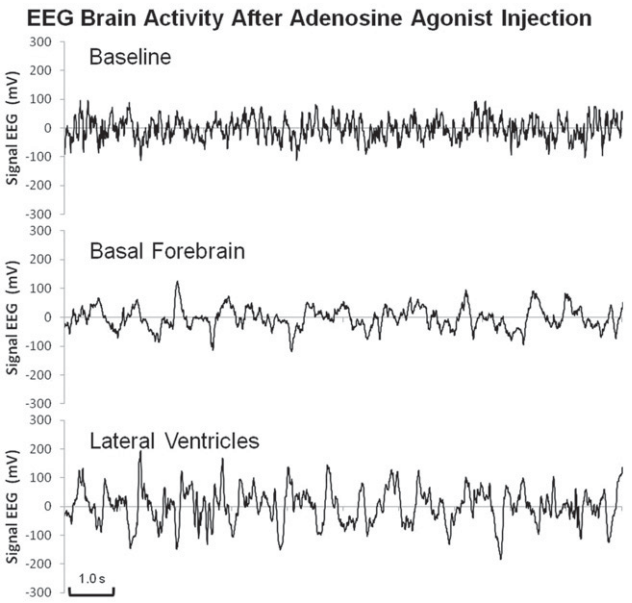

Figure 1. Cortical EEG tracings before adenosine injection (baseline) and 1 h after adenosine agonist microinjections at MTDs for two different locations. The A1 agonist CPA was delivered ICV to the LVs at a dose of 700 μg or was injected directly into the BF at a dose of 350 μg. The desynchronous EEG activity (low amplitude, high frequency) of the baseline indicates wakefulness. The adenosine agonists produced signals with higher amplitude and lower frequencies; the EEG responses are similar to deep sedation.

While the adenosine agonists were administered centrally, there appeared to be some peripheral side-effects such as a reduction in cardiovascular output and development of pallor, particularly in the LVs group. Since only peripheral and not central AR1 stimulation had been shown to have cardiovascular effects (Schindler et al., 2005), a fraction of the CPA likely escaped the CNS and entered peripheral circulation. In contrast to the LVs group, the BF group maintained their pink skin and mucous membrane coloration.

After transcardial perfusion and fixation, the brains were sectioned and stained with Nissl. A trained pathologist then analyzed the sections and verified that CPA treatment was non-toxic and that cannula placement was grossly accurate. For most animals, cannulae were implanted accurately, and there were no signs of infection beyond what was to be expected after an aseptic surgical procedure. However, three LVs rats and two BF rats developed moderate to severe infections from the surgery or had inaccurate cannulae placements. They were removed from the study.

Adenosine agonist to LVs after soman challenge

CPA protected the animals from soman-induced seizure and convulsions. Figure 2 shows EEG tracings from an animal that received CPA treatment and another animal that received multisol after exposure to soman. It illustrates CPA's suppression of soman-induced spike activity. As shown in Table 4(A), the 12 control animals that did not receive CPA but multisol exhibited seizure onset times of 7.2 ± 3.1 min on an average after exposure to a $1.6 \times LD_{50}$ soman challenge. All 12 control animals also developed convulsions (motor toxic sign = 3) as well as a prostrated posture (general toxic sign = 3). None of the animals that received 700 µg CPA via ICV developed seizure. They were deeply sedated and were not responsive to any external stimuli, similar to the animals that received CPA during the MTD tests. There were no convulsions or behavioral signs of a cholinergic crisis throughout the 5-h monitoring period. Furthermore, there were no indications of a central excitotoxicity; the EEG data resembled that of deep non-random eye movement sleep. In addition to silent neuronal activity, those treated with CPA experienced mild hypothermia. Their temperatures decreased over the 5-h period from a baseline of 37 °C to 32.8 ± 1.3 °C. That decrease in temperature after treatment was significantly different from the results in the control group, which had an average temperature of 37.2 ± 0.8 °C after 5 h ($p < 0.01$).

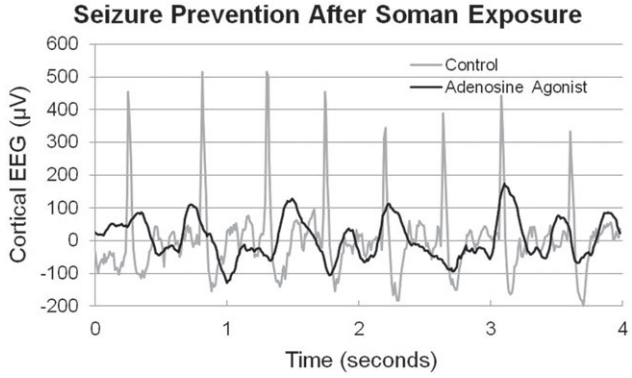

Figure 2. The effects of CPA delivered via ICV on neuronal activity after soman exposure. Thirty minutes after HI-6 (125 mg/kg, IP) pretreatment, animals were exposed to a $1.6 \times LD_{50}$ dose (180 µg/kg, SC) of soman. One minute following soman exposure, the treated animal received AMN (2 mg/kg, IM) and the A1R agonist CPA at MTD (700 µg, ICV). The control animal received multisol instead of CPA. Whereas the control animal developed excitotoxic brain activity (gray tracing), the CPA-treated animal was protected and did not seize (black tracing).

CPA's protection from soman-induced seizure and convulsion continued over-night. Table 5(A) displays survival and neuropathology data for the LVs group. Out of 12 animals, 10 animals that received CPA survived to the 24-h time point and did not show signs of toxicity. Eight of the 10 treated animals were awake and aware of their surroundings; the other two were still in a sleep-like state at the 24-h time point.

Table 4. The effects of adenosine agonists on soman-induced seizure onset time and toxic motor signs.

	Number seize	Minutes to seizure onset, mean ± standard deviation	Toxic motor sign, mean ± standard deviation
(A) CPA or multisol vehicle injected into the lateral ventricles (LVs)			
Control	12/12	7.2 ± 3.1	3 ± 0
CPA: LVs	0/12* ($p < 0.01$)	No seizure activity* ($p < 0.01$)	0 ± 0* ($p < 0.01$)
(B) CPA or multisol vehicle injected into the basal forebrain (BF)			
Control	11/12	9.1 ± 2.0	2.9 ± 0.3
CPA: BF	6/12 ($p = 0.07$)	20.2 ± 14.7* ($p < 0.01$)	1.9 ± 1.2* ($p < 0.01$)

Rats received CPA into the LVs (4A) and the BF (4B) 1 min after exposure to a $1.6 \times LD_{50}$ dose (180 µg/kg) of soman. Latency to seizure was recorded and toxic motor signs scored. A toxic motor sign of 0 indicates normal behavior, whereas a score of 3 designates convulsions (see Table 2). Statistical differences between treatment and control groups' seizure rates were calculated using the Chi-square test, seizure onset using the Kaplan–Meier survival analysis and log rank test, and toxic motor signs using a generalized linear model. Statistically significant differences are indicated by *$p < 0.05$.

Table 5. The effects of adenosine agonists on soman-induced lethality and neuropathology scores at 24 h.

		Neuropathology – group mean ± standard deviation				
	24-h survival	Piriform	Thalamus	Dorsal hippocampus	Ventral hippocampus	Total
(A) CPA injected into the lateral ventricles (LVs)						
Control	1/12	4	3	3	3	13
CPA: LVs	10/12* ($p < 0.01$)	2 ± 0.7†	0.5 ± 0.5†	1.2 ± 0.8†	1.2 ± 0.6†	4.9 ± 1.5†
(B) CPA injected into the basal forebrain (BF)						
Control	9/12	3.57 ± 1.1	2.86 ± 1.3	3.14 ± 1.2	3.57 ± 1.1	13.1 ± 4.6
CPA: BF	6/12 ($p = 0.55$)	1.7 ± 1.9 ($p = 0.1$)	1.3 ± 1.5 ($p = 0.13$)	1.7 ± 1.9 ($p = 0.17$)	1.7 ± 1.9 ($p = 0.1$)	6.3 ± 7.0 ($p = 0.13$)

Twenty-four hours after soman exposure and adenosine treatment, animals that survived were perfused and prepared for histology. A trained neuropathologist graded 4 of the brain regions for damage: the piriform cortex, thalamus, dorsal, and ventral hippocampus. A score of 0 indicates no damage, 4 indicates severe damage in each brain area. Pathology scores for the treatment group were not significantly different from the control according to the Mann–Whitney test ($p > 0.05$). The Kaplan–Meier survival analysis and log rank test were used to analyze differences in survival 24 h after exposure. ICV treatment produced a significant improvement compared with the control whereas the difference between BF treatment and controls were not significantly different.

*Statistically different responses between treatment and control groups.

†LV histological statistical significance could not be determined with $N = 1$ for control.

Only one of the 12 control animals survived 24 h and was mildly uncoordinated (general toxic sign = 1) at that time. Applying Fisher's exact test, the number of surviving animals in the treated group was significantly higher than in the control group ($p < 0.01$). The neuropathology for that control animal indicated moderate to severe damage in the four assessed areas for a total score of 13 (0 = normal, 16 = most severe damage). The CPA-treated animals on an average had a pathology score of 4.9 ± 1.5 [$N = 10$]. Although the substantial reduction in brain pathology was not significantly different from the control group (due to control, $N = 1$), the LVs treatment pathology scores were significantly ($p < 0.01$) better than the BF controls (Table 5B).

Adenosine agonist CPA to the BF after soman challenge

The animals that received CPA to the BF as treatment for soman exposure experienced variable protection; seizure and motor response results are reported in Table 4(B). Unlike the LV group that had 100% seizure prevention, six of the 12 animals (50%) in the BF treatment group were protected from seizure. According to Fisher's exact test, that the rate of seizure prevention is not statistically significant ($p = 0.07$). Those protected animals displayed sleep-like behavior similar to the LV group. Although six treated animals did go into seizure, their latency to seizure onset (20.2 ± 14.7 min) was delayed compared with the control group (9.1 ± 2.0 min). When all animals from the treated and control groups are compared, the difference in seizure latency is statistically significant ($p < 0.01$). One of the 12 control animals did not develop EEG seizure activity but did display signs of soman poisoning: impaired movement, tremors, and convulsions. Three of the six CPA-treated animals that did not seize produced no peripheral signs of toxicity. The other three treatment animals did eventually develop mild tremors approximately 30–45 min after soman exposure. The difference between seizure onset and time of death were statistically different between the BF and LV treatment groups according to the Kaplan–Meier survival analysis and log rank test ($p < 0.05$). The difference in minimum body temperatures between the CPA-treated ($34.3 \pm 2.3\,^{\circ}C$ [$N = 12$]) and control BF ($36.4 \pm 1.0\,^{\circ}C$ [$N = 12$]) groups was statistically significant according to the t test ($p < 0.05$).

The 24-h survival and neuropathology results for the BF group are described in Table 5(B). Six of the 12 animals treated with CPA survived to the 24-h time point, four of which did not seize the day before. The BF multisol control group experienced better survivability than the LVs; nine of the BF control animals survived to the next day. While the BF-treated group experienced greater lethality than the LV-treated group (6/12 versus 2/12), the localized stimulation of BF ARs provided neuroprotection in addition to preventing seizure. Treated animals that did not seize had zero to minimal neuropathology; their total pathology scores were 0, 0, 4, and 4. Figure 3 illustrates how CPA treatment can protect the brain from neuronal loss if seizures are prevented with either LV (Figure 3, middle) or BF (Figure 3, right) treatments. Similar to what was observed in the control animals that seized, those treated with CPA and seized also developed severe brain damage; their total pathology scores were 14 and 16. Overall, the pathology scores between the BF control and BF treatment groups were not statistically significant ($p = 0.11$). The difference between LV treatment and BF treatment groups also was not statistically different according to the Mann–Whitney test ($p = 0.66$).

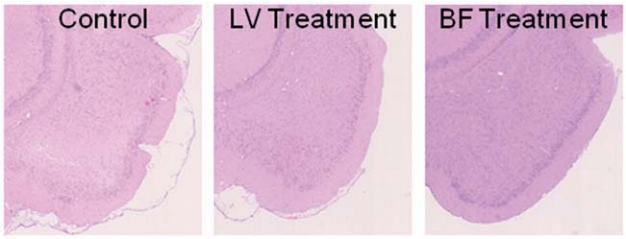

Figure 3. Analysis of the control and treated groups' histology illustrated that CPA administered ICV and to the BF has neuroprotective capabilities. Three example sections containing the piriform cortex (objective 5×) from a control rat (left), an LV treated rat (middle), and BF treated rat (right) are shown. Whereas the control rat displayed a significant decrease in neuronal density, brain tissue in both LV and BF treated rats was preserved.

Discussion

This project investigated new neural pathways for protecting the brain from seizure and neuropathology after nerve agent poisoning. We hypothesized that excitotoxic brain activity could be suppressed by (1) widespread stimulation of A1Rs with CPA delivered ICV, or (2) focal stimulation of BF A1Rs with direct CPA microinjections. We believed that the greatest potential for neuroprotection would be achieved when the maximum numbers of ARs were stimulated. Therefore, the first objective was to determine the MTDs for each treatment regimen. After determining the MTDs, both general and focal AR stimulations were tested in a soman seizure rat model with success; seizures and neuropathology were reduced with adenosine agonist treatment. Although direct brain injections of a treatment are not feasible outside the laboratory, this work demonstrated that central AR stimulation is a promising therapeutic mechanism for countering nerve agent neuropathology.

General stimulation of central ARs with CPA microinjected ICV provided the most consistent protection against a soman challenge. The prevention of a central cholinergic crisis can largely be attributed to adenosine's pre- and post-synaptic actions. A1R pre-synaptic stimulation inhibits the release of ACh and glutamate, thereby limiting the accumulation of excitatory neurotransmitters in the synaptic cleft. A1R post-synaptic stimulation further suppresses neuronal activity and decreases Ca^{++} influx. Both these actions may block nerve agent-induced hyper-excitatory activity that typically leads to neuropathology. This link between seizure prevention and neuroprotection is supported by the significant reduction in total neuropathology scores from the severe to minimal.

Focal adenosine agonist treatment to the BF displayed mixed results. While some treated animals were completely protected from seizure or convulsion, others had the same

reaction to soman that the control animals did. These variable results are consistent with the data collected from the dose escalation procedure for determining the MTD. While some animals were profoundly affected by CPA microinjected into the BF, others in that same group may have had very little reaction. The most probable explanation for this variation is inconsistent sites of microinjection. The basal forebrain is a very small target to hit accurately, even with a computer-assisted stereotaxic procedure. Differences between animal shape, size and skull features exacerbate the likelihood for the placement of the cannulae outside the effective region. Deviation in expected responses during the MTD tests may also be due to the desensitization of AR stimulation after the first day of testing. Because of this effect, future dose-determination studies should extend recovery times or only inject once per animal.

Focal BF AR stimulation was not expected to mitigate soman's lethal peripheral effects. Since the current therapeutic strategies are able to manage the peripheral but not the cholinergic crisis, the objective for this intervention was to prevent seizure and suppress neuropathology. The treatment group was, therefore, as susceptible to soman's lethal peripheral effects (e.g. hyper-secretion) as the control group. Consequently, survival rates were not expected to be significantly different. To improve survival rates in addition to providing neuroprotection, BF AR stimulation should be combined with therapeutic doses of atropine and/or an AChE reactivator.

Since ICV-delivered CPA demonstrates more consistent anti-seizure and neuroprotective effects, general stimulation of central ARs is a more promising therapeutic approach than targeting local BF ARs. The differences in survival time and seizure onset between LV and BF treatment groups were statistically different but the neuropathology scores were not. Although focal BF AR stimulation was less effective, the BF should remain a candidate therapeutic target. It is a known source of significant cholinergic activity (Semba, 2000), and in this study, its inhibition protected some animals from neuropathology. Perhaps pharmacologics with BF selectivity or alternative techniques such as optogenetics could be implemented to better evaluate the protective efficacy of BF inhibition by itself or in conjunction with other therapeutic mechanisms.

Previous research investigating the therapeutic benefits of adenosine agonists was confounded by the peripheral side-effects such as bradycardia and hypotension (Bueters et al., 2003; Joosen et al., 2004; van Helden et al., 1998). Those authors concluded that adenosine's neuroprotective benefit was likely caused by the concomitant decline in cardiac output which decreased the amount of nerve agent that circulated to the brain. To establish that neuroprotection can be achieved by the stimulation of central A1Rs, we directly injected adenosine agonists into the brain. While we cannot make any conclusive statements regarding centrally administered adenosine agonists' effect on cardiac output, it is certain that the nerve agent circulated to the brain; histology did not indicate ischemia, and the mucus membranes and tissue were perfused with blood. Furthermore, the EEG data collected from soman exposed and non-exposed animals showed a significant decrease in cortical neuronal activity within 1–2 min after CPA administration. While it is possible that cardiac depression may have played a role in delaying the cholinergic crisis, the results from this study suggest that CPA's fast acting suppression of neurotransmitter release and neuronal inhibition was the primary neuroprotective mechanism. Further studies that measure cardiac output, determine central AChE activity, and record cortical EEG are needed to better understand the effects of cardiac depression in this neuroprotective mechanism.

One observable side-effect of CPA treatment is the steady decrease in body temperature from 37 °C to approximately 28–30 °C over a period of 5 h. Published data suggest this to be a centrally mediated response, driven in a large part by the nucleus of the solitary tract (Tupone et al., 2013). The effect that this decrease in body temperature has on seizure and pathology prevention is unknown. However, hypothermic conditions are known to affect cellular activity (Geeraerts & Vigue, 2009). In some instances, the change in cellular activity can be beneficial, particularly for cardiac trauma and brain injury (McIntyre et al., 2003; Peterson et al., 2008; Schwartz et al., 2012). Furthermore, decreasing body temperature has been shown to suppress seizure activity (D'Ambrosio et al., 2013; Liu et al., 1993). Consequently, adenosine's seizure prevention capacity may not be fully attributable to a decrease in neurotransmitter release or post-synaptic inhibition. It is, therefore, necessary to conduct additional studies where the body temperature is a controlled variable so that the neuroprotective mechanism can be better understood.

Although central AR stimulation is a promising medical countermeasure to nerve agent exposure, direct brain injections of adenosine agonists are not clinically feasible; drugs need to be administered intramuscularly in the field. To begin translating this therapy, future research needs to investigate alternative administration and dosing protocols. Experiments should test if the same neuroprotective benefits can be elicited when CPA is injected systemically. To prevent negative peripheral side effects while maintaining the positive central effects, an adenosine antagonist that is impermeable to the blood–brain barrier could be co-administered. Joosen et al. (2004) investigated that approach and concluded that the peripherally acting antagonist reversed the therapeutic benefit of CPA (Joosen et al., 2004). However, brain EEG data were not collected in that study. It is possible that the antagonist only aggravated peripheral cholinergic symptoms yet central excitotoxic activity continued to be inhibited and the brain was protected (Joosen et al., 2004). Further research into administration methods and side-effect mitigation with EEG monitoring is needed to elucidate this phenomenon. To enhance survivability in addition to providing neuroprotection, greater doses of atropine and AChE reactivators are recommended for suppressing the nerve agent's lethal peripheral effects.

Conclusions

The results from this study support the hypothesis that central AR stimulation helps to suppresses hyper-excitatory and toxic

neuronal activity in a soman-induced seizure rat model. While the pre- and post-synaptic effects are believed to be the primary mechanism of neuroprotection, AR-induced cardiac depression may have contributed to the neuroprotective effect by delaying the circulation of nerve agent to the brain. Additional studies disentangling these two factors are necessary before the mechanism of protection can be truly identified. Although the specific mechanism remains to be established, central AR stimulation via ICV microinjection of CPA provided consistent neuroprotection; seizures were prevented in all exposed animals. In addition to widespread central AR stimulation, this study investigated basal forebrain AR stimulation as a new mechanism for seizure prevention. Focal microinjection of CPA into the basal forebrain either delayed or prevented seizure onset in the soman model. Although 100% seizure prevention is the objective, the finding that seizure was completely prevented in six of the 12 animals suggests the cholinergic BF is a promising therapeutic target. Improved outcomes from BF stimulation may be achieved with more precise BF delivery, refined dosing regimens, and alternative pharmacological formulations. The positive results from this study motivate further research into this therapeutic approach. Future work aims to elucidate the neuroprotective mechanism and to address delivery and efficacy limitations.

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Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper. The authors also gratefully acknowledge the financial and administrative support that the ARL provided.

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References

Anderson R, Sheehan MJ, Strong P. (1994). Characterization of the adenosine receptors mediating hypothermia in the conscious mouse. Br J Pharmacol 113:1386–90.

Basheer R, Strecker RE, Thakkar MM, Mccarley RW. (2004). Adenosine and sleep-wake regulation. Prog Neurobiol 73:379–96.

Benington JH, Kodali SK, Heller HC. (1995). Stimulation of A1 adenosine receptors mimics the electroencephalographic effects of sleep deprivation. Brain Res 692:79–85.

Biaggioni I. (1992). Contrasting excitatory and inhibitory effects of adenosine in blood pressure regulation. Hypertension 20:457–65.

Bjorness TE, Greene RW. (2009). Adenosine and sleep. Curr Neuropharmacol 7:238–45.

Bowen SE, Balster RL. (1997). A comparison of the acute behavioral effects of inhaled amyl, ethyl, and butyl acetate in mice. Fundam Appl Toxicol 35:189–96.

Bueters TJ, Groen B, Danhof M, et al. (2002). Therapeutic efficacy of the adenosine A1 receptor agonist N6-cyclopentyladenosine (CPA) against organophosphate intoxication. Arch Toxicol 76:650–6.

Bueters TJ, Joosen MJ, Van Helden HP, et al. (2003). Adenosine A1 receptor agonist N6-cyclopentyladenosine affects the inactivation of acetylcholinesterase in blood and brain by sarin. J Pharmacol Exp Ther 304:1307–13.

Compton JR. (2004). Adenosine receptor agonist Pd 81 723 protects against seizure/status epilepticus and neuropathology following organophosphate exposure. Published by WRAIR and available through DTIC. Silver Spring (MD): Walter Reed Army Institute of Research.

Cunha RA. (2005). Neuroprotection by adenosine in the brain: from A(1) receptor activation to A (2A) receptor blockade. Purinergic Signal 1: 111–34.

D'ambrosio R, Eastman CL, Darvas F, et al. (2013). Mild passive focal cooling prevents epileptic seizures after head injury in rats. Ann Neurol 73:199–209.

Dixon AK, Gubitz AK, Sirinathsinghji DJ, et al. (1996). Tissue distribution of adenosine receptor mRNAs in the rat. Br J Pharmacol 118:1461–8.

Dunwiddie TV, Masino SA. (2001). The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci 24:31–55.

El-Etri MM, Nickell WT, Ennis M, et al. (1992). Brain norepinephrine reductions in soman-intoxicated rats: association with convulsions and AChE inhibition, time course, and relation to other monoamines. Exp Neurol 118:153–63.

Fosbraey P, Wetherell JR, French MC. (1990). Neurotransmitter changes in guinea-pig brain regions following soman intoxication. J Neurochem 54:72–9.

Geeraerts T, Vigue B. (2009). Cellular metabolism, temperature and brain injury. Ann Fr Anesth Reanim 28:339–44.

Gouder N, Fritschy JM, Boison D. (2003). Seizure suppression by adenosine A1 receptor activation in a mouse model of pharmacoresistant epilepsy. Epilepsia 44:877–85.

Haas HL, Selbach O. (2000). Functions of neuronal adenosine receptors. Naunyn Schmiedebergs Arch Pharmacol 362:375–81.

Harrison PK, Bueters TJH, Ijzerman AP, et al. (2003). Partial adenosine A1 receptor agonists inhibit sarin-induced epileptiform activity in the hippocampal slice. Eur J Pharmacol 471:97–104.

Huber A, Guttinger M, Mohler H, Boison D. (2002). Seizure suppression by adenosine A(2A) receptor activation in a rat model of audiogenic brainstem epilepsy. Neurosci Lett 329:289–92.

Joosen MA, Bueters TH, Helden HM. (2004). Cardiovascular effects of the adenosine A1 receptor agonist N6-cyclopentyladenosine (CPA) decisive for its therapeutic efficacy in sarin poisoning. Arch Toxicol 78:34–9.

Joosen MJ, Van Helden HP. (2007). Correlations between acetylcholinesterase inhibition, acetylcholine levels and EEG changes during perfusion with neostigmine and N6-cyclopentyladenosine in rat brain. Eur J Pharmacol 555:122–8.

Lallement G, Carpentier P, Collet A, et al. (1991). Effects of soman-induced seizures on different extracellular amino acid levels and on glutamate uptake in rat hippocampus. Brain Res 563:234–40.

Lin JS, Anaclet C, Sergeeva OA, Haas HL. (2011). The waking brain: an update. Cell Mol Life Sci 68:2499–512.
Liu Z, Gatt A, Mikati M, Holmes GL. (1993). Effect of temperature on kainic acid-induced seizures. Brain Res 631:51–8.
Lynge J, Hellsten Y. (2000). Distribution of adenosine A1, A2A and A2B receptors in human skeletal muscle. Acta Physiol Scand 169:283–90.
Malva JO, Silva AP, Cunha RA. (2003). Presynaptic modulation controlling neuronal excitability and epileptogenesis: role of kainate, adenosine and neuropeptide Y receptors. Neurochem Res 28:1501–15.
Mcdonough Jr JH, Dochterman LW, Smith CD, Shih TM. (1995). Protection against nerve agent-induced neuropathology, but not cardiac pathology, is associated with the anticonvulsant action of drug treatment. Neurotoxicology 16:123–32.
Mcdonough JH, Shih T-M. (1997). Neuropharmacological mechanisms of nerve agent-induced seizure and neuropathology. Neurosci Biobehav Rev 21:559–79.
Mcintyre LA, Fergusson DA, Hébert PC, et al. (2003). Prolonged therapeutic hypothermia after traumatic brain injury in adults: a systematic review. JAMA 289:2992–9.
Mesulam MM, Mufson EJ, Levey AI, Wainer BH. (1983). Cholinergic innervation of cortex by the basal forebrain: cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (*Substantia innominata*), and hypothalamus in the rhesus monkey. J Comp Neurol 214:170–97.
Methippara MM, Kumar S, Alam MN, et al. (2005). Effects on sleep of microdialysis of adenosine A1 and A2a receptor analogs into the lateral preoptic area of rats. Am J Physiol Regul Integr Comp Physiol 289:R1715–23.
O'donnell JC, Acon-Chen C, Mcdonough JH, Shih TM. (2010). Comparison of extracellular striatal acetylcholine and brain seizure activity following acute exposure to the nerve agents cyclosarin and tabun in freely moving guinea pigs. Toxicol Mech Methods 20:600–8.
O'donnell JC, Mcdonough JH, Shih TM. (2011). *In vivo* microdialysis and electroencephalographic activity in freely moving guinea pigs exposed to organophosphorus nerve agents sarin and VX: analysis of acetylcholine and glutamate. Arch Toxicol 85:1607–16.
Paxinos G, Watson C. (2009). The rat brain in stereotaxic coordinates: compact. 6th ed. San Diego (CA): Elsevier, Academic Press.
Peterson K, Carson S, Carney N. (2008). Hypothermia treatment for traumatic brain injury: a systematic review and meta-analysis. J Neurotrauma 25:62–71.
Ribeiro JA, Sebastiao AM, De Mendonca A. (2002). Adenosine receptors in the nervous system: pathophysiological implications. Prog Neurobiol 68:377–92.
Schindler CW, Karcz-Kubicha M, Thorndike EB, et al. (2005). Role of central and peripheral adenosine receptors in the cardiovascular responses to intraperitoneal injections of adenosine A1 and A2A subtype receptor agonists. Br J Pharmacol 144:642–50.
Schubert P, Ogata T, Marchini C, et al. (1997). Protective mechanisms of adenosine in neurons and glial cells. Ann NY Acad Sci 825:1–10.
Schwartz BG, Kloner RA, Thomas JL, et al. (2012). Therapeutic hypothermia for acute myocardial infarction and cardiac arrest. Am J Cardiol 110:461–6.
Semba K. (2000). Multiple output pathways of the basal forebrain: organization, chemical heterogeneity, and roles in vigilance. Behav Brain Res 115:117–41.
Shih TM. (1982). Time course effects of soman on acetylcholine and choline levels in six discrete areas of the rat brain. Psychopharmacology (Berlin) 78:170–5.
Shih TM. (1990). Anticonvulsant effects of diazepam and MK-801 in soman poisoning. Epilepsy Res 7:105–16.
Shih TM, Duniho SM, Mcdonough JH. (2003). Control of nerve agent-induced seizures is critical for neuroprotection and survival. Toxicol Appl Pharmacol 188:69–80.
Shih TM, Hulet SW, Mcdonough JH. (2006). The effects of repeated low-dose sarin exposure. Toxicol Appl Pharmacol 215:119–34.
Shih TM, Koviak TA, Capacio BR. (1991). Anticonvulsants for poisoning by the organophosphorus compound soman: pharmacological mechanisms. Neurosci Biobehav Rev 15:349–62.
Sperlagh B, Vizi ES. (2011). The role of extracellular adenosine in chemical neurotransmission in the hippocampus and Basal Ganglia: pharmacological and clinical aspects. Curr Top Med Chem 11: 1034–46.
St Hilaire C, Carroll SH, Chen H, Ravid K. (2009). Mechanisms of induction of adenosine receptor genes and its functional significance. J Cell Physiol 218:35–44.
Strecker RE, Morairty S, Thakkar MM, et al. (2000). Adenosinergic modulation of basal forebrain and preoptic/anterior hypothalamic neuronal activity in the control of behavioral state. Behav Brain Res 115:183–204.
Svenningsson P, Hall H, Sedvall G, Fredholm BB. (1997). Distribution of adenosine receptors in the postmortem human brain: an extended autoradiographic study. Synapse 27:322–35.
Thakkar MM, Delgiacco RA, Strecker RE, Mccarley RW. (2003). Adenosinergic inhibition of basal forebrain wakefulness-active neurons: a simultaneous unit recording and microdialysis study in freely behaving cats. Neuroscience 122:1107–13.
Tuovinen K. (2004). Organophosphate-induced convulsions and prevention of neuropathological damages. Toxicology 196:31–9.
Tupone D, Madden CJ, Morrison SF. (2013). Central activation of the A1 Adenosine Receptor (A1AR) induces a hypothermic, torpor-like state in the rat. J Neurosci 33:14512–25.
Van Helden HP, Groen B, Moor E, et al. (1998). New generic approach to the treatment of organophosphate poisoning: adenosine receptor mediated inhibition of ACh-release. Drug Chem Toxicol 21: 171–81.
Wade JV, Samson FE, Nelson SR, Pazdernik TL. (1987). Changes in extracellular amino acids during soman- and kainic acid-induced seizures. J Neurochem 49:645–50.
Wardas J. (2002). Neuroprotective role of adenosine in the CNS. Pol J Pharmacol 54:313–26.
Yildirim M, Marangoz C. (2007). Anticonvulsant effects of focal and intracerebroventricular adenosine on penicillin-induced epileptiform activity in rats. Brain Res 1127:193–200.
Young D, Dragunow M. (1994). Status epilepticus may be caused by loss of adenosine anticonvulsant mechanisms. Neuroscience 58: 245–61.
Youssef AF, Santi BW. (1997). Simple neurobehavioral functional observational battery and objective gait analysis validation by the use of acrylamide and methanol with a built-in recovery period. Environ Res 73:52–62.

Appendix

Functional observation battery score sheet

Paw/ear color:

0 Pink, normal
1 Pale
2 Blue

Startle reflex:

Stimulus: Snap your fingers close to and above the rodent's head

0 jumps, seems startled
1 more energetic response than two – may include vocalization
2 slight reaction, ear flick, or some evidence that a sound was heard
3 no reaction

Righting reflex:

0 normal (immediately rights itself)
1 slightly impaired (>1 s)
2 impaired (>2 s)
3 totally impaired (remains on back)

Animal handling (ease of handling rodent in hand):

0 Difficult, squirming, twisting, attempting to bite, with, or without vocalizations
1 Moderately easy; vocalizations, little, or no squirming
2 Easy, but alert, limbs may be pulled against body
3 Easy, but lethargic

Arousal:

0 Normal (alert, exploratory movements)
1 Somewhat low (some exploratory movements with periods of immobility)
2 Low (some head or body movement)
3 Very low (little or absent)

Gait description:

0 Normal
1 Impairment uncoordinated movement (ataxia), walking on toes, splayed hind limbs, exaggerated hind limb flexion, staggered gait, dragging hind limbs, unable to walk
2 No movement

Approach response:

Stimulus: slowly use pen to approach the rodent from the front and make sure they are aware of the approach.

0 energetic response, possible vocalizations
1 slow approach, sniffing or turning away
2 no reaction